thereby initiating a rapid change in the fumarate/succinate ratio, the free radical concentration increases to a maximum and then declines to zero

Our results suggest, therefore, that the free radicals observed in heart particles arise from equilibrium processes catalyzed by succinic dehydrogenase and that their steady-state concentration is governed by the parameters which affect this equilibrium. The paper which follows presents quantitative data on the relation between these parameters and the free radical concentration, which confirm the foregoing conclusion and characterize the reaction mechanisms involved in free radical formation.

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*FREE RADICALS IN HEART MUSCLE MITOCHONDRIAL PARTICLES: MECHANISM OF FORMATION**

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A preceding paper[1] describes electron spin resonance (ESR) experiments on the free radicals associated with the succinoxidase system of heart particles. The results indicate that the free radicals arise from redox equilibria involving succinate and one or more components of the succinoxidase system which function on the reducing side of the site of Antimycin A inhibition. Of these components, succinic dehydrogenase, a flavin enzyme[2, 3] appears to be involved specifically in free radical formation.

In the succinoxidase system, succinic dehydrogenase mediates electron transport between the system's ultimate electron donor, succinate, and the rest of the electron transport system, possibly reacting specifically with cytochrome *b*.[4-6] In view of the ESR data described in the preceding paper, the observed free radicals might arise in either or both the coupled oxidation-reduction of the enzyme's prosthetic group with succinate and fumarate, or with a component of the electron transport system functioning on the reducing side of the site of Antimycin A inhibition.

The present paper describes experiments designed to specify the origin of the succinoxidase free radicals more precisely. In these experiments the effects of variations in the concentrations of succinate, fumarate, malonate, and heart parti-

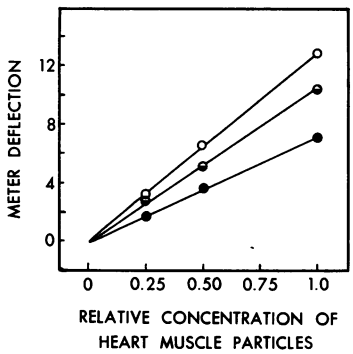


FIG. 1.—Effect of dilution of heart particles contained in a closed glass cell on the ESR signals from anaerobic systems containing fumarate and succinate. Open circles, half solid circles and solid circles represent, respectively, fumarate plus succinate concentrations of 50×10^{-3} *M*, 20×10^{-3} *M* and 2×10^{-3} *M*. The fumarate/succinate mole ratio in each case is 2. The ordinate represents the meter deflection due to the ESR signal at the magnetic field at which the deflection is at a maximum. This value is proportional to the free radical concentration. All points are corrected for any resonance in glass of cell and represent the average of 3 to 9 values.

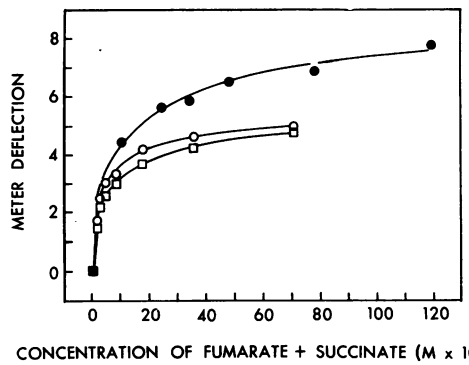


FIG. 2.—Effect of the total concentration of fumarate plus succinate on the ESR signal from heart particles under anaerobic conditions in closed glass cell. Open circles: Fumarate/succinate mole ratio of 2. Open squares: Fumarate/succinate mole ratio of 0.1. Solid circles: Fumarate/succinate mole ratio of 1 and different heart particle preparation. Ordinate as in Fig. 1. All points are corrected for the dilution of heart particles and any resonance in glass of cell and represent the average of 3 to 7 values.

cles on the observed free radical concentration have been determined. These data lead to conclusions which serve to characterize the reaction mechanism responsible for the appearance of free radicals in the heart particle succinoxidase system.

Experimental Methods.—The techniques employed in these studies were the same as those described in the preceding paper.[1] Fumarase assays were made by observing changes in the optical density at 205 mμ in systems containing buffer, heart particle preparation, and 10^{-4} *M* fumarate, all at pH 7.4. Reaction and reference cuvettes were identical with the exception of the presence of fumarate in the reaction cuvette. Free radical concentration was estimated from the maximum meter deflection exhibited by the ESR signals.

Results.—Figure 1 shows the effect of the concentration of heart particles under

anaerobic conditions on the ESR signals at different total concentrations of succinate plus fumarate. The intercepts are (0, 0) and the concentrations of free radicals bears a linear relationship to the concentration of heart particles.

Figure 2 illustrates the effect of the total concentration of fumarate plus succinate on the ESR signal from heart particles under anaerobic conditions. The curves appear to have a (0, 0) intercept and their slopes everywhere decrease and approach zero slope asymptotically. These characteristics suggest a quadratic relationship between free radical concentration and the total concentration of fumarate plus succinate. The effect of the mole ratio of fumarate to succinate on the ESR signal in anaerobic systems of heart particles is illustrated in Figures 3*a* and 3*b*. It is evident that the size of the ESR signal and therefore the free radical

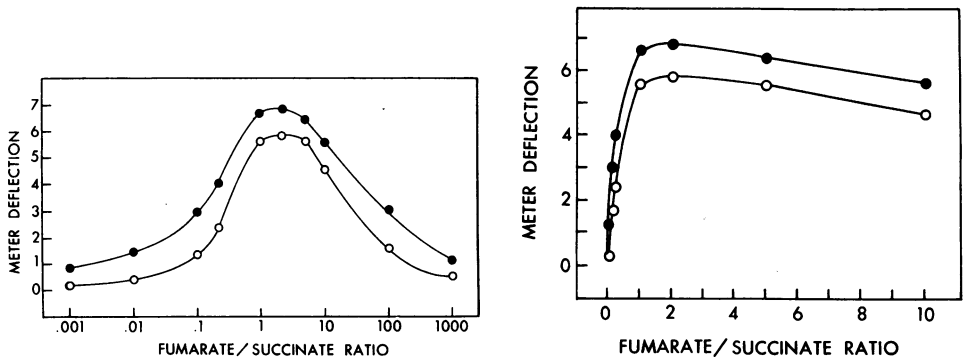


FIG. 3.—Effect of the mole ratio of fumarate to succinate under anaerobic conditions in a closed glass cell on the ESR signal from heart particles at different total concentrations of succinate and fumarate. Figure 3*a*: semilog plot. Figure 3*b*: linear coordinates. Solid circles: 47.7×10^{-3} *M* fumarate plus succinate. Open circles: 24.4×10^{-3} *M* fumarate plus succinate. Ordinate as in Fig. 1. All points are corrected for the dilution of heart particles and for any resonance in glass of cell and represent the average of 3 to 6 values.

concentration is markedly affected by the fumarate to succinate ratio. A maximum free radical concentration is observed when the fumarate succinate mole ratio is about 1.7. The position of this maximum is not affected by a 15-fold difference in total concentration of succinate and fumarate between 3×10^{-3} *M* and 5×10^{-2} *M*. On either side of this maximum, the free radical concentration declines, approaching zero when succinate or fumarate are present alone. Below the maximum the slope everywhere increases as seen in Figure 3*b*. A flex point lies above the maximum since the curve approaches zero asymptotically. The shape and limit characteristics of the curves of Figure 3*b* suggest a cubic relationship between free radical concentration and fumarate to succinate ratio.

Figure 4 illustrates the effect of malonate concentration on the ESR signals from heart particles under anaerobic conditions. The slope of the curve is everywhere negative and increases with increasing concentrations of malonate. The slope approaches zero asymptotically at zero free radical concentration. These data indicate a quadratic relationship between free radical concentration and malonate concentration.

The heart particle preparations show no detectable fumarase activity. This means that in the foregoing experiments fumarase-catalyzed destruction of fumarate does not occur, so that the concentration of fumarate remains unchanged with time.

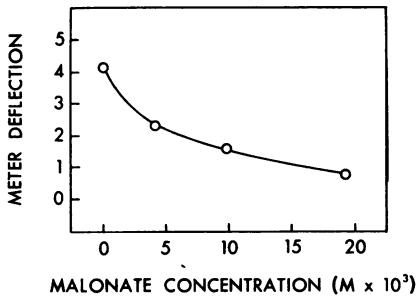
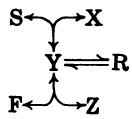


FIG. 4.—Effect of malonate on the ESR signals from heart particles in the presence of fumarate and succinate under anaerobic conditions in closed glass cell. Fumarate/succinate mole ratio of 2; 19.6×10^{-3} M in fumarate plus succinate. Ordinate as in Fig. 1. All points are corrected for the dilution of heart particles and any resonance in glass of cell and represent the average of 3 to 7 values.

Discussion.—Data reported in the previous paper[1] show that the magnitude of the ESR signal, and therefore the free radical concentration, observed in heart particles depends on the presence of succinate and the activity of succinic dehydrogenase and is reversibly dependent on temperatures at least between 0° and 35°C. The data presented above relate to the reversible interactions among the concentrations of free radicals, fumarate, succinate, malonate, and heart particles under conditions which preclude net electron transport. The results support the conclusion that the free radicals observed arise as the result of the redox equilibria involving succinate, fumarate, and the enzyme, succinic dehydrogenase, which catalyzes the redox equilibrium between these two compounds. Any mechanism proposed to explain the existence of the free radicals, therefore, must involve as a minimal requirement the participation of succinate, fumarate, and the oxidized and reduced forms of succinic dehydrogenase. Such a mechanism must also satisfy the various function relationships and limit conditions indicated by the data.

In what follows we shall examine several possible biochemical mechanisms and consider whether their properties conform to the requirements imposed by the data described above. Of the schemes considered, Mechanisms 1 and 2 satisfy these minimal requirements and, at the same time, are not inconsistent with what is known about the biochemistry of succinic dehydrogenase. Mechanism 2, which considers the involvement of enzyme-substrate complexes, is more probable than Mechanism 1 from a biochemical point of view.[7, 8]

Mechanism 1:



Here X, Z, S, F, Y and R represent respectively the concentrations of oxidized and reduced succinic dehydrogenase, succinate, fumarate, reaction intermediate, and free radical. The following equilibria are involved:

$$\frac{FZ}{SX} = K_1, \frac{Y}{SX} = K_2, \frac{Y}{FZ} = K_3, \text{ and } \frac{Y}{R} = K_4.$$

Let

$$F/S = Q$$

$X + Y + Z + R = T$, total succinic dehydrogenase concentration, and

$$F + S = C.$$

By solving for R in terms of the measurable variables C, T, and Q one obtains:

$$R = \frac{K_2CTQ}{K_4Q^2 + Q(K_4K_1 + K_4 + K_2K_4C + K_2C) + K_4K_1} \quad (1)$$

In this expression the free radical concentration, R, is cubic with respect to Q, quadratic with respect to C, and linear with respect to T. In these respects it conforms to the data described above. The limit and intercept properties of equation (1) also satisfy the data. A plot of R_{CT} vs Q exhibits a maximum. It can be shown that at the maximum, $Q = \sqrt{K_1}$, and therefore the position of the maximum is independent of C and T, as the data suggest. As Q approaches 0, $\left(\frac{\partial R}{\partial Q}\right)_{CT}$ approaches K_2CT/K_4K_1, that is, $\left(\frac{\partial R}{\partial Q}\right)_{CT}$ is finite and positive. As Q approaches ∞, $\left(\frac{\partial R}{\partial Q}\right)_{CT}$ approaches 0 from negative values. These properties are exhibited by the experimental curves in Figures 3*a* and 3*b*.

The properties of curves which relate R_{QT} to C are of interest in that they offer the justification for including in Mechanism 1 the equilibrium: $R \rightleftharpoons Y$, where $Y/R = K_4$. As C approaches ∞, R_{QT} approaches $T/(K_4 + 1)$. It is apparent that if Y equals zero, that is, if this equilibrium did not exist, K_4 would equal zero and the limit of R would be simply T. Physically this would mean that where the total concentration of substrate, C, is high nearly all the succinic dehydrogenase would exist as the free radical. We offer no proof that this would occur. Therefore, Mechanism 1 is offered as a more general case where this remarkable consequence need not be considered.

The effects of malonate may be treated in the following manner. It is assumed that, since malonate competes with succinate,[9] the inhibitor may interact with oxidized succinic dehydrogenase, X, but not with the reduced enzyme, Z. A fifth equilibrium is then introduced into Mechanism 1: $M + X \rightleftharpoons N$, where $N/MX = K_5$ and M and N represent, respectively, the concentrations of malonate and the complex formed between malonate and oxidized succinic dehydrogenase. Now T, total succinic dehydrogenase concentration, equals $X + Y + Z + R + N$. By solving for R with respect to Q, C, T, and M, one obtains the expression:

$$R = \frac{K_2CQT}{M(Q^2K_4K_5 + QK_4K_5) + K_4Q^2 + Q(K_4K_1 + K_4 + K_2K_4C + K_2C) + K_1K_4} \quad (2)$$

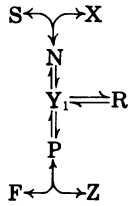
which at constant Q, C, and T, assumes the form:

$$R = \frac{a}{bM + c}$$

R is quadratic in M just as the data would seem to require. As M approaches ∞, R approaches 0. As M approaches 0, R approaches a/c, i.e. equation (2) reduces to equation (1). Similar results also are obtained if one considers that malonate competes with both succinate and fumarate[8-10] for the enzyme.

In Mechanism 1 the involvement of enzyme-substrate complexes was not specifically considered. These are taken into account in Mechanism 2.

Mechanism 2:



Here X, Z, S, F, and R have the same meaning as above. In addition, N and P are enzyme-substrate complexes, and Y_1 is a reaction intermediate with a physical significance related to that of Y in Mechanism 1. The following equilibria are sufficient for our discussion:

$$\frac{FZ}{SX} = K_1, \frac{N}{SX} = K_{10}, \frac{Y_1}{N} = K_{11}, \frac{P}{FZ} = K_{12}, \frac{Y_1}{P} = K_{13}, \text{ and } \frac{Y_1}{R} = K_{14}.$$

It is assumed that $S \gg N$ and $F \gg P$ so that $S + F = C$, as in Mechanism 1. Q has the same meaning as before, but now $T = X + Z + Y_1 + R + N + P$.

By solving R with respect to C, Q, and T, one obtains an expression which is identical with equation (1) with the following exceptions: K_2 and K_4 in equation (1) are replaced by $(K_{10}K_{11} + K_{10} + K_{10}K_{11}/K_{13})$ and $(K_{14} + K_{14}/K_{11} + K_{14}/K_{13})$, respectively. The consequences of Mechanism 2 are the same as those of Mechanism 1. The only essential difference is that the terms Y_1, N, and P are included together in the term Y of Mechanism 1 and the constants K_{10}, K_{11}, K_{13}, and K_{14} are included in K_2 and K_4 as indicated.

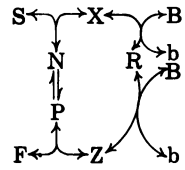
Similar results are obtained if one omits Y_1 from Mechanism 2, leaving the equilibria $N \rightleftharpoons R \rightleftharpoons P$ instead. Under this arrangement K_{11}, K_{13}, and K_{14} become zero and one must consider the expressions $N/R = K_{15}$ and $P/R = K_{16}$. Now K_2 and K_4 in Equation *1* are replaced by $(K_{10} + K_{10}K_{16}/K_{15})$ and $(K_{15} + K_{16})$, respectively.

Mechanisms 1 and 2 represent two of the simplest mechanisms which are consistent with the ESR data and with present knowledge concerning the catalytic function of succinic dehydrogenase. In both of these mechanisms the free radicals arise solely as redox intermediates in the equilibrium among succinate, fumarate, and succinic dehydrogenase, all of which are regarded as 2-electron donor-acceptors. However, other classes of mechanisms are biochemically feasible. For example, free radicals could conceivably arise from an interaction between succinic dehydrogenase and a 1-electron donor-acceptor, such as a cytochrome. Inhibitor studies described in the preceding paper show that the free radicals observed in the heart particle system are formed by reactions which lie on the reducing side of the site of Antimycin A inhibition. The only known components of the succinoxidase system which can be reduced aerobically by succinate in the presence of Antimycin A are succinic dehydrogenase and cytochrome *b*.[4, 5] There is some evidence that succinic dehydrogenase can react directly with cytochrome *b* in heart particles.[4–6, 11, 12] For these reasons we shall consider, in what follows, the possibility that the observed free radicals arise from interactions between the 2-electron donor-

acceptor (succinic dehydrogenase) and a 1-electron donor-acceptor (such as cytochrome *b*).

Consider the possibility that succinic dehydrogenase interacts with a 1-electron donor-acceptor in the manner shown in Mechanism 3.

Mechanism 3:



Here S, F, X, Z, R, N, and P have the same meaning as defined above. The terms b and B refer to the oxidized and reduced forms of the 1-electron donor-acceptor, respectively. In this case let $\beta = B + b$ and $T = X + Z + R + N + P$. It is assumed that β/T is constant in heart particles. In this mechanism, the free radical does not result directly from interactions among succinate, fumarate, and succinic dehydrogenase, but rather from interactions among this enzyme and the one electron donor-acceptor by way of bimolecular reactions. The free radical, R, here may be taken to represent the semi-reduced form of succinic dehydrogenase.

When one solves for R in terms of the experimental variables C, Q, and T, the resultant expression does not have characteristics required by the data. The most striking inadequacy involves the relationship between R_{TQ} and C. It can be shown that, while the relationship is quadratic, R_{TQ} approaches zero asymptotically as C approaches ∞. R_{TQ} is finite when C is zero. This relationship is entirely different from that depicted in Figure 2. When one considers a simpler variation of Mechanism 3 where the enzyme-substrate complexes, N and P, are neglected, the expression for R becomes independent of C. This relationship is also contradictory to the experimental results.

As indicated by the foregoing discussion, it is possible to determine whether the observed relationships between the magnitude of the ESR signal and the parameters which affect it conform with the properties of various proposed reaction mechanisms. The manner in which free radical concentration depends on substrate concentrations leads to the general conclusion that only those mechanisms in which free radicals occur solely as intermediates in an equilibrium involving succinate, fumarate, and succinic dehydrogenase, conform with the observed results. Hence, in the heart particle system, we can exclude specifically as a possible source of the observed free radicals, the equilibrium redox interactions of succinic dehydrogenase with (*a*) 1-electron donor-acceptors, such as cytochromes, and (*b*) any 2-electron agents except, of course, the substrates, fumarate, and succinate.

If the above conclusions are correct it should be possible to calculate from the ESR data the over-all equilibrium constant, K_1, for the reaction: succinate + oxidized succinic dehydrogenase $\rightleftharpoons$ fumarate + reduced succinic dehydrogenase. From the equilibrium equations which describe Mechanisms 1 and 2, it can be shown that $Q^2_{R\ \max} = K_1$, where $Q_{R\ \max}$ is the fumarate/succinate ratio which yields a maximum free radical concentration (or ESR signal). The value (1.72) of $Q_{R\ \max}$ ascertained from the data of Figure 3*a* yields a value of 2.95 for K_1. From this value one can calculate the free energy, $\Delta F°$, and the redox potential, $\Delta E'_0$,

for the reaction. This leads to a value of -670 cal. mole^{-1} for ΔF°, and $+.015$ volts for $\Delta E'_0$ at pH 7.4 and 35°C.

$\Delta E'_0$ is defined as the difference between the E'_0 for the enzyme redox couple (reduced enzyme $\rightleftharpoons$ oxidized enzyme $+ 2H^+ + 2e$) and the E'_0 for the substrate couple (succinate $\rightleftharpoons$ fumarate $+ 2H^+ + 2e$). Since E'_0 for the succinate-fumarate couple, which is known from potentiometric data, is $0.00v$ at pH 7,[13] E'_0 for the enzyme couple is $+.015v$ at this pH. It will be noted that this value lies between the E'_0 for the fumarate-succinate couple ($0.00v$) and the E'_0 for the cytochromes which occur in the succinoxidase system ($+0.1$ to $0.3v$).[14-18] This relationship is to be expected from the known sequence of electron transport in this system: substrate-enzyme-cytochromes. That the value of E'_0 for the enzyme, independently determined from ESR data, conforms with this expectation, tends to confirm our conclusions regarding the reaction mechanism responsible for the observed free radicals.

These considerations lead to the following conclusions regarding the free radicals observed in heart particles in the presence of succinate: (1) The free radicals arise in the equilibrium among succinate, fumarate, and succinic dehydrogenase. (2) The free radicals constitute one or more particular electron configurations of a complex composed of succinic dehydrogenase and its substrate. (3) The free radicals do not arise from the reaction between succinic dehydrogenase and redox systems which, in the succinoxidase system, are capable of reoxidizing the reduced enzyme. Specifically the data rule out the origin of free radicals in the reaction between succinic dehydrogenase and either 1-electron systems such as cytochrome *b* or 2-electron systems such as quinones or another flavin.

Although our data do not further specify the electron configuration of the dehydrogenase-substrate complex which is responsible for the observed ESR signal, certain possible arrangements merit discussion.

In the formation of the free radical enzyme-substrate complex, two molecules, in which all electrons occur in pairs, combine to form a structure which gives rise to an ESR signal and must therefore contain one or more unpaired electrons. One possible mechanism is the formation, within the complex, of a biradical containing two unpaired electrons. The ESR signal yielded by such a structure will depend on the location of the unpaired electrons within the complex. Generally, unpaired electrons separated by about 4A° or less will interact sufficiently to yield an ESR signal so broad as to escape detection in the spectrometer.[19]

When the separation is sufficient to minimize interaction, the resultant signal may be of two types. If the two unpaired electrons occur in similar atomic environments, at least with respect to those nuclei capable of magnetic interaction with the electron (e.g., *H*, *N*), they may yield identical ESR signals, which superimpose to form the observed signal. If the atomic environments of the two electrons are distinctive, two separable signals, of approximately equal intensities, should result. The ESR signal observed in the heart particles shows no evidence that it is comprised of two equal signals.[1] A possible explanation of the observed results is, therefore, that the enzyme-substrate complex contains two unpaired electrons which are in equivalent atomic environments, and which do not interact appreciably with each other.

An alternative explanation takes into account the possible mediation of electron

transfer by the iron atoms which occur in succinic dehydrogenase.[8] The enzyme molecule contains 4 iron atoms, two of which are firmly bound.[8, 16] Singer and coworkers [10, 20] have suggested a reaction mechanism in which it is supposed that the iron atoms may mediate electron exchange among succinate, fumarate, and the flavin prosthetic group within the enzyme-substrate complex of succinic dehydrogenase. In this case the following equilibrium may occur: (Succinate-Flavin-Fe^{+++}-Fe^{+++}) $\rightleftharpoons$ (Fumarate-Flavin-Fe^{+++}-Fe^{++})* $\rightleftharpoons$ (Fumarate-Flavin H_2-Fe^{+++}-Fe^{+++}) $\rightleftharpoons$ (Fumarate-Flavin-Fe^{++}-Fe^{++}). (In these and subsequent configurations, the asterisk denotes an unpaired electron.) In this scheme an intermediate form of the enzyme-substrate complex, (Fumarate-Flavin-Fe^{++}-Fe^{+++})*, contains one unpaired electron and will exhibit an ESR signal typical of an organic free radical. This proposal is also consistent with our experimental observations.

It is significant that the ESR data rule out the occurrence of detectable concentrations of free radicals in reactions involving succinic dehydrogenase and a 1-electron donor-acceptor. A possible reaction of this type is the redox interaction between succinic dehydrogenase and cytochrome *b*.[4-6] One of the chief arguments introduced by Michaelis[21] in his original proposals that free radicals must occur as redox intermediates is that they provide a reasonable mechanism for coupling a 2-electron system (such as succinic dehydrogenase) to a 1-electron system (such as a cytochrome). It is of interest, therefore, to consider possible explanations for the absence of a detectable free radical in this reaction.

Here too, the proposal of Singer *et al.*[16, 20] regarding the possible participation of iron atoms in succinic dehydrogenase activity is pertinent. These workers propose,[10] on the basis of inhibitor and oxidant specificity studies,[8, 20] that ferrous iron atoms exist in the reduced enzyme so that the prosthetic group may be represented as (Flavin-Fe^{++}-Fe^{++}). It is believed that the existence of ferrous atoms in the prosthetic group enables the reduced enzyme to interact efficiently with 1-electron oxidants. The oxidation of the reduced enzyme by 1-electron oxidants may be supposed to occur in two successive 1-electron-1-electron reactions to form (Flavin-Fe^{++}-Fe^{+++}) as intermediate and (Flavin-Fe^{+++}-Fe^{+++}), which represents oxidized succinic dehydrogenase, as the final product. Neither the intermediate nor the final product is a free radical, as required by our ESR data. Our data indicate that possible free radical intermediates of the succinic dehydrogenase prosthetic group, such as (Flavin)* or (Flavin-Fe^{+++}-Fe^{+++})*, do not exist in detectable concentrations in the heart particle system. (See Mechanism 3.)

It is worth noting that apart from evidence regarding the participation of free radicals in enzyme reactions, ESR data are also capable of yielding reaction parameters not easily attainable by other means. The peculiar spectral properties of succinic dehydrogenase[20] do not permit an accurate determination of the ratio of oxidized to reduced enzyme in the presence of succinate and fumarate. In the absence of this information, neither the equilibrium constant for the reaction between the enzyme and its substrate, nor the redox potential of the enzyme itself can be calculated. As shown above, the ESR data lead to reasonable estimations of these values, even though the enzyme is present as part of the complex electron transport system, and no spectrophotometric data on its oxidation state can be obtained.

The foregoing results suggest certain generalizations regarding the circumstances which govern the occurrence of detectable concentrations of free radicals in oxidation-reduction enzyme systems. In this respect the most relevant outcome of the above experiments is that the ESR signal associated with the succinic dehydrogenase enzyme system in heart particles is due to a free radical form of an enzyme-substrate complex. This conclusion leads to the generalization that factors which affect the concentration of this complex will govern the intensity of the ESR signal.

This generalization may be employed to account for most of the results of the ESR experiments on redox enzyme systems that have been reported thus far which include the necessary kinetic observations.

For an enzyme system in an equilibrium condition, the following relationships hold: oxidized enzyme + reduced substrate $\rightleftharpoons$ enzyme-substrate complex $\rightleftharpoons$ reduced enzyme + oxidized substrate. Accordingly, the concentration of the enzyme-substrate complex is a function of the product: [oxidized enzyme][reduced substrate] or of the product: [reduced enzyme][oxidized substrate]. As pointed out in the earlier discussion of Mechanisms 1 and 2, the concentration of the enzyme-substrate complex will go through a maximum at a particular reduced substrate/oxidized substrate (or oxidized enzyme/reduced enzyme) ratio depending, among other things, on the difference between the redox potentials of the enzyme and substrate redox couples. Where the difference is small, the ratio at which the maximal concentration of the complex occurs will be close to 1. If the difference in redox potentials is large, the required ratio becomes considerably smaller or larger than 1. According to the reaction described in Mechanisms 1 and 2, the free radical form of the enzyme-substrate complex is in equilibrium with the ordinary form of the complex. The free radical concentration is therefore proportional to the concentration of the ordinary enzyme-substrate complex, the proportionality constant being, presumably, characteristic for a given enzyme system.

Hence, for an enzyme system in equilibrium conditions, the concentration of free radical intermediate, and therefore the intensity of the observed ESR signal will depend on the variable factors which govern the concentration of the enzyme-substrate complex: (1) the absolute concentrations of enzyme and substrate and (2) the oxidized enzyme/reduced enzyme ratio. The free radical concentration will also depend on two invariant factors, which are characteristics for a given enzyme: (3) the affinity of the enzyme for its substrate and (4) the proportionality constant for the equilibrium between the ordinary form of the enzyme-substrate complex, and its free radical form.

Only when the ratios between oxidized and reduced enzyme and substrates in an equilibrium system are such as to yield a maximal concentration of the free radical-substrate-enzyme complex will a relatively intense ESR signal be observed. Since these ratios remain constant in equilibrium conditions, the signal will persist at a constant intensity as long as the enzyme remains unaffected by the experimental conditions. This situation is typified by the succinic dehydrogenase of the heart particle system in conditions of zero electron transport.[1]

Two types of situations may occur in enzyme systems that are in non-equilibrium conditions and undergo net oxidation or reduction. The distinction depends on how close to equilibrium the system maintains itself as the reaction progresses. If the system is such that the reaction progresses with the system nearly in equi-

librium, the free radical concentration will be governed by the same parameters which are controlling in the case of true equilibrium, i.e., the reactant concentrations, and the oxidized enzyme/reduced enzyme ratio. An experimental test for this state is that the free radical concentration is not altered significantly when equilibrium conditions are imposed on a non-equilibrium system. An example of this case is the heart particle system under aerobic conditions. When oxygen is excluded so that equilibrium conditions are established, no significant change in the size of the ESR signal is observed. (See Fig. 4 in the preceding paper[1].)

In a non-equilibrium enzyme system in which there is a considerable difference between the rates of the forward and reverse reactions, the concentration of the enzyme-substrate complex, and of its free radical form, will depend on factors which govern the rate of the predominant reaction. In general the concentration of enzyme-substrate complex will show a linear dependence on enzyme concentration and a saturation with increasing concentration of the substrate for the predominant reaction. A test for this state is the observation that the free radical concentration varies significantly when conditions leading to equilibrium are imposed on a non-equilibrium system. An example is the earlier investigation of lactic oxidative decarboxylase[22, 23] which showed that the size of the ESR signal is proportional to the enzyme concentration, and increases to a maximum with increasing lactic acid concentration. In this case the ESR signal is proportional to the net rate of oxidation.

On the basis of these criteria it is probable that in earlier ESR experiments with the cytochrome reductase system[24] and the old yellow enzyme system,[25] essentially equilibrium conditions were controlling while non-equilibrium conditions prevailed in the case of the alcohol dehydrogenase system.[24]

These considerations show that an ESR signal due to a free radical form of the complex between a redox enzyme and its substrate may exhibit a wide range of relationships to the net oxidative activity of the system, depending on the specific thermodynamic conditions which govern the process. A corresponding variety may be expected among the situations encountered in ESR investigations of intact living cells.

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THE SUSTAINED GROWTH OF HUMAN AND ANIMAL CELLS IN A PROTEIN-FREE ENVIRONMENT

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Although a few strains of mammalian cells have been grown in a protein-free medium,[1–7] most of the cultures now available require protein, usually added as whole or dialyzed serum. The role of that protein is not clear. It has been found to promote the adhesion of cells to a glass surface, and their subsequent flattening;[8, 9] but this is not its only or perhaps even primary function, for it is also required by cells growing in suspension. Since the protein amino acids are utilized to only a minor degree for the synthesis of cell protein,[10] it seemed possible that it might be providing one or more essential compounds of small molecular weight, either initially bound to the protein, or formed by its degradation. This was borne out by the present experiments, which show that protein as such is not required for the growth of the human or animal cells so far studied. Its primary function is to provide as yet unidentified compound(s), sufficiently small to pass through a cellophane membrane, and the provision of which is promoted by the addition of a pancreatic extract.

Methods.—The dual culture apparatus used in most of these experiments was a modification of that used by Nurmikko[11] and by McLimans *et al.*,[12] and consisted of two cylindrical culture vessels, each with a horizontal side tubulation terminating in a ground glass flange. Sterile silicone grease was spread on each flange, a cellophane membrane made from $^{24}/_{32}''$ Visking clear cellophane tubing was interposed, and the two vessels were then joined. The cell culture in each cylinder was kept dispersed by a suspended and free-spinning magnetic bar. The courtesy of Dr. William F. McLimans in providing a prototype model in advance of publication is greatly appreciated. The cell lines used were human strains KB and HeLa, a cloned subculture of HeLa (S3), and a mouse fibroblast, strain L 929. The suspension cultures used as inoculum were grown in a minimal basal medium containing the 28 demonstrably essential growth factors[13] supplemented with 5 per cent dialyzed human or horse serum.